Biochem. J. (1980) **192**, 951–954
Printed in Great Britain

Mitochondrial and cytosolic ATP/ADP ratios in isolated hepatocytes

A comparison of the digitonin method and the non-aqueous fractionation procedure

Sibylle SOBOLL, Theodorus P. M. AKERBOOM, Wolfgang-Dietrich SCHWENKE, Roland HAASE and Helmut SIES
Institut für Physiologische Chemie I, Universität Düsseldorf, Moorenstrasse 5, D-4000 Düsseldorf, Germany

(*Received 14 August 1980/Accepted 4 September 1980*)

The ratio of ATP content/ADP content in the mitochondrial matrix was found to be 2.07 ± 0.21 and 2.26 ± 0.22 as determined with six different preparations of isolated hepatocytes subfractionated with the digitonin and non-aqueous-fractionation procedures, respectively. In contrast, the mitochondrial matrix ATP/ADP determined with isolated haemoglobin-free perfused liver by using the non-aqueous-fractionation procedure was about 0.2, whereas the cytosolic values obtained with isolated cells and with the intact organ were similar. It is concluded that the relatively higher ATP/ADP ratio in the mitochondrial matrix of isolated hepatocytes represents a biochemical difference due to properties of the model rather than a methodological artifact.

In recent years, several techniques have been developed for the study of intracellular compartmentation (for a review, see Akerboom *et al.*, 1979). A major parameter of interest in the investigation of metabolic aspects of bioenergetics has been the ATP/ADP ratio and, related to this, the phosphorylation state, $[ATP]/[ADP][P_i]$, in mitochondrial matrix and cytosol (for a review, see Bücher & Sies, 1980). Although similar cytosolic ATP/ADP ratios of about 8.0 were obtained with the digitonin method originated by Zuurendonk & Tager (1974) and with the non-aqueous-fractionation procedure developed by Elbers *et al.* (1974), a marked difference exists with regard to the mitochondrial matrix values. These are approx. 0.2 with the latter technique as applied to the haemoglobin-free perfused liver of fed rats (Soboll *et al.*, 1978), but ATP/ADP ratios of about 2 were obtained with the digitonin method as applied to incubations of isolated hepatocytes (Zuurendonk & Tager, 1974; Siess & Wieland, 1976; Sies *et al.*, 1977; Akerboom *et al.*, 1978).

It was argued by Soboll *et al.* (1978) on the basis of experiments with isolated mitochondria (Heldt & Klingenberg, 1968) that phosphorylation of mitochondrial ADP will occur during digitonin fractionation even at 0°C in isolated hepatocytes, so that the high values obtained have to be considered as an artifact. Although metabolic controls (Akerboom *et al.*, 1978; Siess *et al.*, 1978; Zuurendonk *et al.*, 1979) and temperature controls (Brocks *et al.*, 1980) support the assumption that phosphorylation of mitochondrial ADP during the fractionation procedure with digitonin is negligibly low, a direct comparison of the two methods seemed desirable.

Experimental

Preparation and incubation of isolated hepatocytes

Hepatocytes were isolated from male Wistar rats (150–180g), fed on stock diet (Altromin), as described by Berry & Friend (1969) with slight modifications (Sies *et al.*, 1977). For incubation, hepatocytes were transferred into 25ml conical flasks containing incubation medium at 37°C, equilibrated with a gas mixture of O_2/CO_2 (19:1, v/v). Final concentrations were: glucose, 10mM; L-lactate, 2.1mM; pyruvate, 0.3mM; DL-3-hydroxybutyrate, 0.6mM; acetoacetate, 0.3mM; L-ornithine, 2.4mM; and the salt mixture described by Krebs & Henseleit (1932). Hepatocytes were added to a final concentration of 30–40mg dry wt./ml. Shaking frequency was $120\,min^{-1}$. Samples were taken after 20min.

Digitonin fractionation

The digitonin-fractionation procedure (Zuurendonk & Tager, 1974) was used exactly with the modification described by Sies *et al.* (1977). The separation medium (5ml) layered on top of a silicone oil mixture (Wacker Chemie AR 200/General Electric SF 96/100; 8:1, v/v) consisted of 0.25M-sucrose/20mM-Mops (4-morpholinepropanesulphonic acid) (pH 7.0)/3mM-EDTA/2mM-

0306-3283/80/120951-04$01.50/1

digitonin, at −2°C, and the exposure time after addition of 1 ml of hepatocyte incubation mixture was 15 s. Lactate and glutamate dehydrogenases were measured as marker enzymes (Sies *et al.*, 1977). Pellet fractions are designated mitochondrial, and supernatant fractions are designated cytosolic.

Non-aqueous fractionation

The procedure as developed for the isolated perfused rat liver (Elbers *et al.*, 1974; Soboll *et al.*, 1978) was modified for isolated hepatocytes as follows. For freeze–stop, approx. 10 ml of the hepatocyte incubation mixture was quickly poured onto the surface of an aluminum block precooled in liquid N_2, and a tablet of 2 m thickness was formed by putting another precooled aluminum block on top. The dimensions of the lower block were: height, 7.6 cm; diameter, 10 cm; with a 1.0 cm ridge of height 1.8 mm on the top. After grinding in liquid N_2 and lyophilization, about 0.1 g of lyophilized liver cells were sonicated in a mixture of heptane/carbon tetrachloride (density 1.23 g/ml) cooled in heptane/solid CO_2, for 4 min, with 5 s periods of sonication and cooling alternately. The homogenate was layered on top of a density gradient of the same solvents from 1.29–1.43 g/ml, and 8–9 fractions were obtained. Citrate synthase and phosphoglycerate kinase were measured as marker enzymes (Soboll *et al.*, 1978). The percentage of adenine nucleotides present in the mitochondrial and cytosolic compartments was calculated by extrapolating the data to fractions of pure mitochondria and pure cytosol (Elbers *et al.*, 1974; cf. Fig. 1). The total contents were calculated by assuming 565 mg of protein/g dry wt.

For comparison, a non-aqueous subfractionation of isolated haemoglobin-free perfused rat liver is also shown in Figs. 1(*c*) and 1(*d*) (cf. Soboll *et al.*, 1978).

Assays

The measurement of ATP, ADP, protein and the marker enzymes was done as previously (Sies *et al.*, 1977; Soboll *et al.*, 1978). Adenine nucleotide assays were performed on a Sigma ZWS 11 dual-wavelength spectrophotometer (Biochem, München, Germany) at 340–400 nm.

Results

Table 1 gives the subcellular distribution of adenine nucleotides in isolated hepatocytes fractionated with the digitonin method and with the non-aqueous fractionation procedure. Clearly, the two methods provide similar results. First, the total contents of ATP and ADP, as well as their ratio, are similar, indicating that the freeze–stop and sub-

Table 1. *Subcellular distribution of adenine nucleotides in isolated hepatocytes fractionated with the digitonin method and with the non-aqueous fractionation procedure*

Isolated hepatocytes were incubated for 20 min as indicated in the Experimental section, and subsequently samples were taken for subfractionation employing the two different procedures. Data from six different preparations are given, ±S.E.M. The results given for the digitonin method represent separate measurements of the total (unfractionated) incubation, the pellet fraction (mitochondrial), and the supernatant fraction (cytosolic). The results given for the non-aqueous fractionation method represent measurements of the total incubation (after freeze–clamp and lyophilization); the mitochondrial and cytosolic values are obtained after fractionation and calculation of the relative contribution following extrapolation to pure fractions (see Fig. 1).

Method	Total			Mitochondrial matrix			Cytosol		
	Content (μmol/g dry wt.) of:			Content (μmol/g dry wt.) of:			Content (μmol/g dry wt.) of:		
	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
Digitonin method	6.23 ± 0.52	1.25 ± 0.10	5.10 ± 0.44	1.22 ± 0.13	0.61 ± 0.07	2.07 ± 0.21	4.80 ± 0.62	0.76 ± 0.06	6.58 ± 0.97
Non-aqueous fractionation method	6.98 ± 0.57	1.60 ± 0.09	4.37 ± 0.32	1.16 ± 0.10	0.53 ± 0.06	2.26 ± 0.22	5.82 ± 0.58	1.07 ± 0.10	5.70 ± 0.66

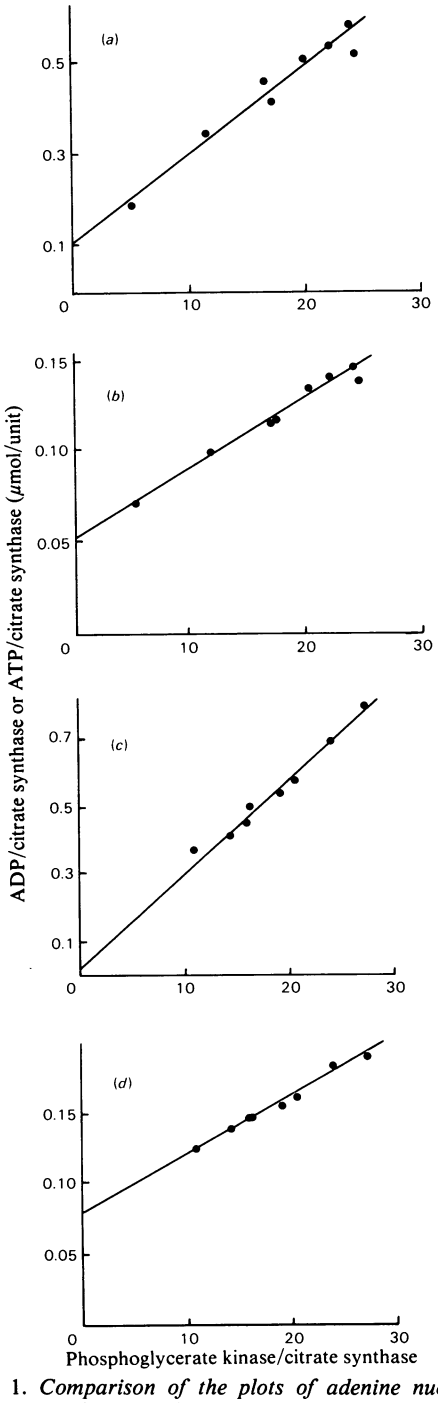

Fig. 1. *Comparison of the plots of adenine nucleotide contents in fractions from non-aqueous subfractionation in density gradients obtained from isolated hepatocytes (a, b) and from haemoglobin-free perfused liver (c, d)*
Data are given as ATP (*a*, *c*) or ADP (*b*, *d*)/citrate synthase ratios on the ordinate versus the ratio of measured marker enzyme activities in the different fractions (phosphoglycerate kinase/citrate synthase) on the abscissa, averaged from six and four gradients for *a* and *b* and *c* and *d* respectively (cf. Elbers *et al.*, 1974; Akerboom *et al.*, 1979). (*a*) and (*b*) are data from a single preparation of isolated hepatocytes. The mitochondrial ATP and ADP contents were calculated to amount to 25 and 44%, respectively, of the total hepatocyte content. Marker enzyme activities were 25 and 446 mU/mg of protein for citrate synthase and phosphoglycerate kinase, respectively. ATP and ADP contents were 10.3 and 3.0 μmol/g of protein in the unfractionated sample. (*c*) and (*d*) are data from a haemoglobin-free perfused liver from a fed rat. The mitochondrial ATP and ADP were calculated to amount to 4 and 55%, respectively, of the total liver content. Marker enzyme activities were 29 and 485 mU/mg of protein for citrate synthase and phosphoglycerate kinase, respectively. ATP and ADP contents were 14.6 and 4.2 μmol/g of protein in the unfractionated sample.

sequent lyophilization leave the contents unaltered. Secondly, and this is the central point of interest, the ratios of ATP content/ADP content in the mitochondrial matrix are 2.07 and 2.26, *i.e.* there is quite good agreement between the two methods. Thirdly, the cytosolic values were 6.58 and 5.70 respectively, being statistically not different.

The novel application of the non-aqueous-fractionation procedure to isolated hepatocytes is demonstrated in Figs. 1(*a*) and 1(*b*) to be successful. In fact, when compared with similar data obtained from perfused liver (Figs. 1*c* and 1*d*), a larger range of the phosphoglycerate kinase/citrate synthase ratios is observed, in particular towards the lower values. Thus, the extrapolation to the intercept of the ordinate is at least as reliable as with the data obtained from the whole liver.

Discussion

Currently, two fractionation procedures are being actively used in studies of subcellular compartmentation. Although ample data are available regarding various metabolites, a direct comparison until now has not been undertaken. In the present work, both methods have been applied on one and the same model system under the same metabolic conditions. The results show that the two methods, operating on entriely different principles, lead to essentially the same values. We draw two conclusions from these results.

First, the similarity of the data obtained with the digitonin and the non-aqueous fractionation techniques establishes the validity of both techniques by mutual support.

Secondly, it is demonstrated that isolated hepatocytes exhibit a ratio of ATP content/ADP content in the mitochondrial matrix approximately one order

of magnitude higher than the corresponding value found with the intact perfused organ. It is, therefore, concluded that the high mitochondrial matrix ATP/ADP ratio in isolated hepatocytes represents a biochemical difference due to properties of the model rather than to a methodological artifact. This conclusion is in agreement with the finding that no difference in mitochondrial ATP/ADP ratios is observed when the digitonin method is carried out at +5°C and −5°C (Brocks *et al.*, 1980).

Although this difference between isolated hepatocytes and the intact perfused organ has been demonstrated by the present study, the underlying reason remains to be established. We maintain, however, that both of these experimental models continue to be useful tools in metabolic studies (Krebs *et al.*, 1974; Tager *et al.*, 1976; Bücher & Sies, 1980).

Expert technical assistance was provided by Maria Gärtner, Sonja Gründel and Brigida Zimmer. This work was supported by the Deutsche Forschungsgemeinschaft, grants So 133/1 and Si 255/3.

References

Akerboom, T. P. M., Bookelman, H., Zuurendonk, P. F., Van der Meer, R. & Tager, J. M. (1978) *Eur. J. Biochem.* **84**, 413–420

Akerboom, T. P. M., Van der Meer, R. & Tager, J. M. (1979) *Methods Life Sci. B* **205**, 1–33

Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520

Brocks, D. G., Siess, E. A. & Wieland, O. H. (1980) *Biochem. J.* **186**, 1–6

Bücher, Th. & Sies, H. (1980) in *Cell Compartmentation and Metabolic Channeling* (Nover, L., Lynen, F. & Mothes, K., eds.), pp. 279–302, Gustav Fischer Verlag, Jena and Elsevier/North-Holland Biomedical Press, Amsterdam

Elbers, R., Heldt, H. W., Schmucker, P., Soboll, S. & Wiese, H. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 378–393

Heldt, H. W. & Klingenberg, M. (1968) *Eur. J. Biochem.* **4**, 1–8

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) in *Regulation of Hepatic Metabolism* (Lundquist, F. & Tygstrup, N., eds.), pp. 726–750, Academic Press, New York and London

Sies, H., Akerboom, T. P. M. & Tager, J. M. (1977) *Eur. J. Biochem.* **72**, 301–307

Siess, E. A. & Wieland, O. H. (1976) *Biochem. J.* **156**, 91–102

Siess, E. A., Brocks., D. G. & Wieland, O. H. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 785–798

Soboll, S., Scholz, R. & Heldt, H. W. (1978) *Eur. J. Biochem.* **87**, 377–390

Tager, J. M., Söling, H. D. & Williamson, J. R. (1976) (eds.) *Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies*, North-Holland, Amsterdam

Zuurendonk, P. F. & Tager, J. M. (1974) *Biochim. Biophys. Acta* **333**, 393–399

Zuurendonk, P. F., Tischler, M. E., Akerboom, T. P. M., Van der Meer, R., Williamson, J. R. & Tager, J. M. (1979) *Methods Enzymol.* **55**, 207–223